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Anti-IgM-Induced Growth Inhibition and Apoptosis Are Independent of Ornithine Decarboxylase in Ramos Cells[1]

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Ornithine decarboxylase (ODC) is a key enzyme involved in polyamine production and is thought to regulate growth and apoptosis in multiple cell systems. A potential link between ODC and growth may involve the action of an oncogene *c-myc* which is thought to transcriptionally regulate ODC. We have examined the involvement of ODC in anti-IgM-induced growth inhibition and apoptosis in Burkitt's lymphoma cells. Inhibitors of ODC such as difluoromethylornithine (DFMO) completely blocked ODC activity, resulting in growth inhibition but not apoptosis. Addition of putrescine, the product of ODC enzymatic action, to Ramos cells had only a minor effect on growth, did not cause apoptosis, did not augment or block anti-IgM-mediated growth inhibition and apoptosis, but did reverse DFMO-mediated growth inhibition. Anti-IgM treatment of Ramos cells, which markedly decreased *c-myc* mRNA and protein, caused a paradoxical increase in ODC mRNA level as well as ODC enzymatic activity and increased cellular levels of putrescine. DFMO and putrescine did not alter *c-myc* mRNA levels directly, nor did they have any affects on anti-IgM-mediated down-regulation of *c-myc* mRNA. TNF-α, which inhibited anti-IgM-mediated apoptosis, did not inhibit either anti-IgM or DFMO-mediated inhibition of growth. These agents were without effect on ODC activity itself or on the anti-IgM-mediated increase in ODC activity. From these studies we conclude that ODC inhibition affects growth but is unrelated to the induction of apoptosis. Both anti-IgM-mediated inhibition of growth and induction of apoptosis are independent of ODC. Thus two distinct pathways for growth regulation are present: one in which ODC and polyamines are important and the other cell surface receptor-mediated (sIg) which is independent of ODC and polyamines. © 1997 Academic Press

Key Words: **anti-Ig; c-*myc*; growth inhibition; apoptosis; ornithine decarboxylase.**

INTRODUCTION

Several investigators have suggested that ornithine decarboxylase is an important enzyme in the regulation of growth [1–7] and may lie on the pathway from c-Myc to the regulation of apoptosis [8–10]. Specifically the model proposes that in myeloid 32D cells, transfection with *c-myc*-expressing plasmids leads to high levels of expression of *c-myc* which is associated with up-regulation of ODC[3] and induction of apoptosis. Transfection with an ODC-expressing plasmid led to the same results. Inhibition of ODC by difluoromethylornithine (DFMO) partially blocked the induction of apoptosis by *c-myc* or ODC-expressing plasmids, thus confirming the involvement of ODC in the apoptosis induction [9, 10].

Other studies have also shown that products of the ODC pathway such as putrescine, spermidine, and spermine may regulate growth and apoptosis [11–18]. Polyamines have been known to facilitate the removal of histone from DNA and to act as a source of positive charges to neutralize nucleic acid negative phosphate charges [19–21]. Polyamines may stabilize RNA secondary structures [22], Z DNA [21, 23], or affect the action of topoisomerases in resolving complex DNA structures [24, 25] and thus may keep the DNA in an open configuration which may be important during DNA replication [16]. However, DNA left in an open configuration is vulnerable to fragmentation [26]. Situations involving DNA nicking and repair may also be affected by polyamines [16, 27]. It has also been reported that excessive levels of polyamines, together with polyamine oxidase, may lead to conditions of cellular toxicity which may in turn lead to cell necrosis [28, 29] or apoptosis [17]. The overall picture that emerges is a complex one. Both up-regulation and down-regulation of polyamine levels may perturb the cell and thus contribute to cell death [11–14, 16, 17, 30].

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[3] Abbreviations used: ODC, ornithine decarboxylase; DFMO, DL-2-difluoromethylornithine; α-MO, α-methylornithine; MGBG, methylglyoxal bis (guanylhydrazone); EBV, Epstein–Barr virus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SAM-DC, S-adenosylmethionine decarboxylase; TNF-α, tumor necrosis factor-α.

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Our own studies have been directed at anti-IgM-induced apoptosis in Burkitt's lymphoma cells. We have shown, as have others, that anti-IgM causes apoptosis in certain Burkitt's lymphoma cells which are EBV-negative. By contrast other Burkitt's lymphoma cells which are EBV-positive seem more resistant to apoptosis induction by anti-IgM [31]. The role of *c-myc* in this process is a complex one. Down-regulation of c-Myc levels leads to growth inhibition and in some cases may cause apoptosis [8, 32]. Moreover *c-myc* antisense oligodeoxynucleotides induce growth inhibition in EBV-positive cells but apoptosis in EBV-negative cell lines, implying that *c-myc* down-regulation alone while necessary for growth inhibition may be insufficient to subsequently achieve apoptosis [31].

We now examine the role of ODC in the overall pathways leading from anti-IgM stimulation of Ramos Burkitt's lymphoma cells to the end points of growth inhibition and apoptosis. Specifically, we have explored the link to ODC using inhibitors, such as DFMO and α-methylornithine (α-MO), and by the addition of polyamines (putrescine, spermidine, and spermine). If ODC lies on the pathway from anti-IgM to apoptosis, then regulation of ODC by anti-IgM would be anticipated. We have therefore examined ODC mRNA levels, ODC enzyme activity, and intracellular polyamine levels following treatment of Ramos cells with anti-IgM. Our results point to ODC-dependent and independent pathways of growth regulation and further imply that ODC activity is not a contributor to anti-IgM-induced apoptosis in Ramos cells.

MATERIALS AND METHODS

Materials

Ramos (CRL 1596) cells and plasmids for GAPDH (57090) and ODC (101651) were obtained from American Type Culture Collection (Rockville, MD). Plasmid for v-*myc* was obtained from transfected *E. coli* clones [33]. Affinity-purified goat anti-human IgM polyclonal antibody was obtained from Organon Teknika Corp. (Durham, NC). AIM-V synthetic medium was from Life Sciences (Gaithersburg, MD) and is a non-serum-containing medium formulated with Dulbecco's modified Eagle medium, nutrient mixture F-12, Hepes buffer, human serum albumin, human transferrin, and recombinant human insulin. RPMI 1640, glutamine solution, and cell culture media were from Irvine Scientific (Santa Ana, CA). Fetal calf serum was from Gemini Bioproducts (Calabasas, CA). [^{3}H]thymidine was from New England Nuclear (NEN, Boston, MA). L-[1-^{14}C]ornithine, and [α-^{32}P]dCTP were purchased from ICN (Costa Mesa, CA). Recombinant human TNF-α was purchased from Genzyme (Cambridge, MA). DL-2-difluoromethylornithine was from Research Biochemicals International (RBI) (Natick, MA). α-Methylornithine, methylglyoxal bis(guanylhydrazone), putrescine, spermidine, spermine, 1,7-heptadiamine, dansylchloride, propidium iodide, and other chemicals were purchased from Sigma Chemical (St. Louis, MO).

Methods

Cell culture and treatment. Ramos cells were cultured in RPMI 1640 medium supplemented with penicillin G (100 units/ml), streptomycin (100 μg/ml), fungizone (0.25 μg/ml), L-glutamine (2 mM), 10% heat-inactivated fetal calf serum, and Hepes (pH 7.4, 10 mM) at 37°C in a humidified 5% CO_2 incubator [31]. Cells grown to log phase (2 to 5 × 10^5/ml) were treated with anti-IgM (10 μg/ml), DFMO (1 mM), or putrescine (250 μM) for specific time periods as described in individual experiments. After harvest, the cells were washed with PBS and stored at −20°C until all treatments were collected or alternatively processed immediately. For those experiments where spermidine (100 μM) or spermine (100 μM) treatments were used AIM-V medium was required. Ramos cells were preconditioned in AIM-V for 1 day before use.

ODC enzyme activity assay. ODC activity was assayed by incubating cell extracts with L-[1-^{14}C]ornithine. Enzymatic action releases $^{14}CO_2$ which is trapped onto a filter and counted [10, 34]. Cells (~5 × 10^6) with various experimental treatments were harvested by centrifugation, washed twice in PBS, and then resuspended in 500 μl of ODC assay buffer (10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 2.5 mM DTT, and 0.2 mM pyridoxal-5-phosphate). The cell suspensions were kept at −20°C until all samples were collected. The cells were lysed by three cycles of freeze-thawing, and cell debris was removed by centrifugation for 5 min at 13,000*g*. Duplicates of 200 μl of cell extract were transferred to a 6-ml glass tube with a rubber stopper top (Kontes, K-8823120). A small center well (Kontes, K 882320) was hung from the middle of the rubber stopper top and a filter disk presoaked with Soluene 350 (Packard, Downers Grove, IL) to trap liberated $^{14}CO_2$ from the reaction was placed in the center of the well. To each cell extract 0.2 μCi of L-[1-^{14}C]ornithine (55 mCi/mmol, ICN) was added and incubated for 30 min at 37°C. Trichloroacetic acid (200 μl; 50% w/v) was injected through the rubber top to stop the reaction. After 30 min, the filters were removed and the radioactivity was determined by scintillation counting. ODC assays were standardized for protein content by using the Bio-Rad protein assay and the specific activity was presented as counts per minute per microgram of protein.

HPLC analysis of dansylated polyamines. Polyamine levels in experimental samples were analyzed by HPLC using C_{18}-reverse-phase columns (Phenomenex, Torrance CA) [35, 36]. Washed cell pellets from 10^7 cells were stored at −20°C until assayed. Cells were lysed with 110 μl protein extraction buffer [50 mM Tris, pH 7.5, 2 mM EDTA, 1 mM EGTA, 150 mM NaCl, 50 mM 2-mercaptoethanol, 0.1% SDS, 1% Triton X-100, 0.5% deoxycholate, plus protease inhibitors leupeptin (10 μg/ml), aprotinin (10 μg/ml), PMSF (100 μg/ml), and Na_3VO_4 (1 mM)]. After 30 min of incubation at 0°C, cell debris was removed by centrifugation and a 5-μl aliquot was withdrawn for protein determination. To the 100 μl of supernatant remaining an equal volume of perchloric acid (PCA, 8% w/v) was added and the polyamines in 200 μl of the PCA soluble fraction were retained. A fixed amount (5 nmol) of 1,7-heptadiamine was added to each sample as an internal standard for quantitation purposes. The polyamines were dansylated by the addition of 200 μl 1.5 M Na_2CO_3 and 300 μl of dansychloride (7 mg/ml in acetone) followed by incubation at 60°C for 1 h in the dark. The solvent was then evaporated for 1 h at room temperature and the modified polyamines were solubilized in toluene and again evaporated until dry under vacuum. The samples were redissolved in 100 μl acetonitrile and filtered through a 0.45 μm filter prior to injection of 20 μl onto the HPLC. Separation and elution of the polyamines were achieved at a flow rate of 1.5 ml/min with step gradients of acetonitrile in water as follows: 20 to 60% over 3 min, 60 to 70% over 3 min, hold at 70% for 1 min, 70 to 80% over 3 min, hold at 80% for 1 min, 80 to 100% over 3 min, hold at 100% for 1 min [35]. Recoveries of putrescine, spermidine, and spermine were calculated relative to the recovery of the 1,7-heptadiamine internal standard.

Concentration standards (e.g., 50, 100, 200, and 400 pmol of putrescine, spermidine, and spermine, mixed or separated) were also prepared at the same time to determine retention times and standard curves.

The eluate was monitored by fluorescence spectroscopy (Perkin-Elmer LS 50B, excitation 365 nm, emission 510 nm, excitation and

emission slits both 10 nm). Putrescine, heptadiamine, spermidine, and spermine peaks were analyzed using Perkin–Elmer fluorescence data manager software. Polyamine amounts were determined from the standard curves and normalized with respect to the protein amounts.

Proliferation assays. Proliferation of cells and inhibition of cell growth were assessed by the incorporation of radiolabeled thymidine and by a colorimetric method for a mitochondrial dehydrogenase activity. Cells ($\sim 2.5 \cdot 10^5$/ml) were cultured in a final volume of 5 ml in a 6-well flat-bottom plate (Costar, Cambridge, MA), and treated with reagent for varying time periods. Triplicate aliquots of 200 μl were analyzed for thymidine uptake and another set of triplicate aliquots of 100 μl were analyzed by a colorimetric assay. The first set was incubated with [^{3}H]thymidine (1 μCi/ml) for 4 h in 96-well plates. The cells were harvested and lysed, and the trichloroacetic acid-precipitable materials were collected onto glass filters and counted [37, 38]. Proliferation of cells was also assessed on the second set of triplicate aliquots by a determination of the cellular conversion of tetrazolium salt into formazan product using an ELISA plate reader according to the manufacturer's instructions (Promega Cell-Titer96 nonradioactive cell proliferation assay kit).

Apoptosis analysis by flow cytometry. Apoptosis was assessed by flow cytometry as reported previously [31, 39]. Washed cell pellets (approximately 1×10^6) were resuspended in 1 ml of 70% ethanol for at least 20 min at −20°C (and can then be stored for up to 1 week). The fixed cells were washed twice and resuspended in PBS at a concentration of 1×10^6 cells/ml. Cells were incubated at 37°C for 30 min and stained with propidium iodide (50 μg/ml) for 5 min and analyzed by flow cytometry within 6 h. The resulting histogram profiles were analyzed for ploidy analysis using PC-LYSYS. The software generates a histogram plot of cells number vs. DNA content (i.e., channel number). Peak identification is carried out using control nuclei derived from resting lymphocytes (single G_0 peak) and from Ramos under normal culture conditions, which gives a characteristic G_0/G_1, S, and G_2/M distribution. The apoptotic peak (A_0) is defined as the peak which occurs in channel numbers lower than 200 (G_0/G_1 peak) as described by others [39].

mRNA determination (Northern blot). The changes in *c-myc* and ODC mRNA levels due to treatment in Ramos cells were analyzed by Northern blot as described before [31]. Total RNA was isolated using the RNA STAT-60 kit (TEL-TEST "B" Inc., Friendswood, TX). RNA pellets were resuspended in 50 to 100 μl Tes (10 mM Tris, 5 mM EDTA, 1% SDS, pH 7.4) and quantitated by UV absorption. Equivalent amounts (25 μg) of total RNA from each sample were analyzed using glyoxal gels as described previously. Samples were analyzed with ^{32}P-labeled probes for *myc*, ODC, or GAPDH. Autoradiograms of the gels were obtained by exposure to Kodak XAR-5 film.

RESULTS

Anti-IgM Induction of ODC Activity

To test whether ODC plays an important role in anti-IgM-mediated control of B cells, we first examined the ODC activity following anti-IgM treatment. The cells were also treated with DFMO, a suicide inhibitor of ODC which completely blocks ODC activity [40]. The results shown in Fig. 1 indicate that anti-IgM causes a transient increase in ODC activity between about 6 and 10 h. DFMO by itself causes a rapid decrease (<1 h) in ODC activity. DFMO also blocks the anti-IgM-mediated increase in ODC activity and does so rapidly.

Putrescine treatment of the cells was also explored. Putrescine is the end product of ODC and can function as a feedback inhibitor of this enzyme [6, 29, 41, 42].

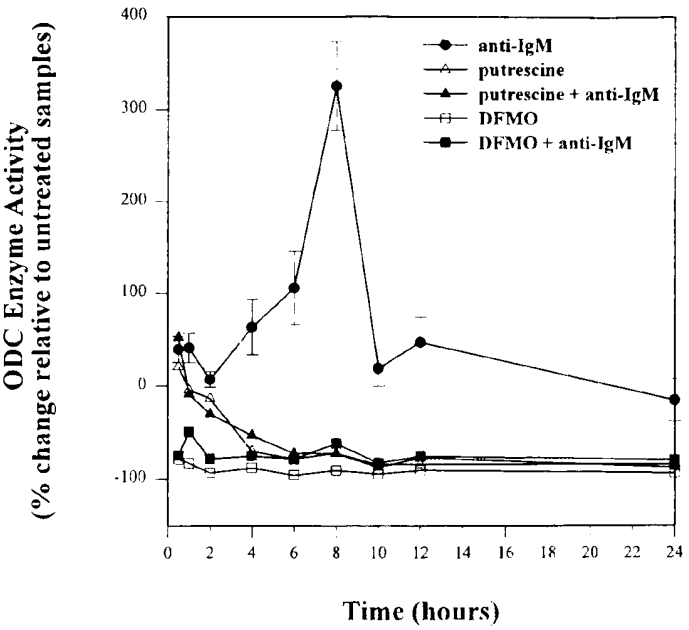

FIG. 1. Effects of anti-IgM or DFMO on ODC enzyme activities. Ramos cells were grown to exponential phase and transferred to 6-well microtiter plates overnight. The cells were treated with either 10 μg/ml anti-IgM, 250 μM putrescine, putrescine plus anti-IgM, 1 mM DFMO, DFMO plus anti-IgM, or no treatment. ODC enzyme activity was then determined at various times as described under Materials and Methods. Results are presented as the percentage change from the values obtained in the untreated samples. Results are the means from four experiments. Error bars (±1 SD) are presented for anti-IgM-treated samples but have been omitted in the others to simplify the presentation.

In agreement with this, putrescine also caused inhibition of ODC activity at about 6 h. In contrast to DFMO which is rapid, the effect of putrescine is slow. Similarly putrescine is a potent but slower acting inhibitor of anti-IgM-meditated increase in ODC activity. Thus anti-IgM up-regulates ODC in a transient manner and this rise in activity is blocked by either DFMO or putrescine. Note should be made of the time course, since ODC activity changes occur fairly late after treatment. These experiments set the stage to test whether changes in ODC activity link anti-IgM treatment to *c-myc* down-regulation, growth inhibition, and apoptosis.

Anti-IgM-Induced Changes in Polyamine Levels

In the previous experiment we have relied on CO_2 production as an assessment of ODC activity and putrescine production. However, the relationship of putrescine to the subsequent metabolites of the pathway (i.e., spermidine and spermine) is complex since all of the polyamines can act as feedback regulators of ODC activity [6, 29, 41, 42] and interconversion between the various polyamines occurs [2, 3]. Because polyamine levels are regulated in complex ways, it is important that the relationship of spermidine and spermine to

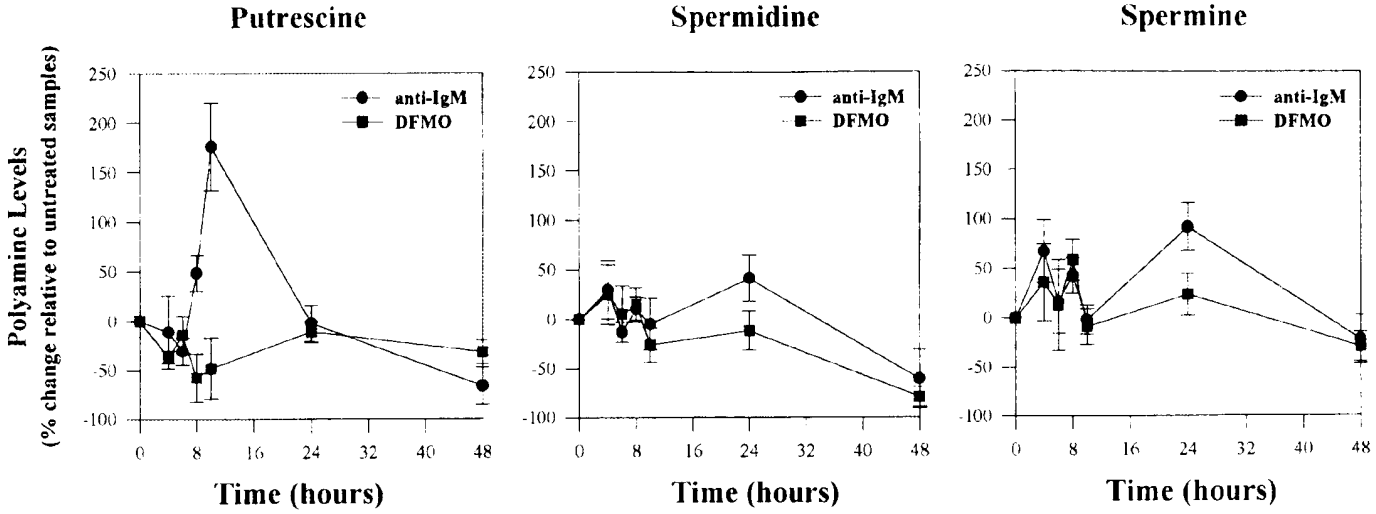

FIG. 2. Effects of anti-IgM or DFMO on polyamine levels. Ramos cells (~1 × 10^7) were transferred to 6-well microtiter plates and cultured overnight. At varying times after addition of either no reagent, 10 μg/ml anti-IgM, or 1 mM DFMO the cells were harvested and intracellular polyamine levels were determined by HPLC as described under Materials and Methods. Polyamine levels (pmol/μg cell protein) were determined and results are presented as percentage change from the values obtained in the untreated samples. Results presented are the means (±1 SD) from three experiments.

putrescine is understood clearly. The cells were therefore treated with buffer, anti-IgM, or DFMO and polyamine levels were determined by HPLC analysis (Fig. 2). Putrescine levels showed a transient rise (about 670 pmol per 10^7 cells) following anti-IgM treatment at times similar to those at which ODC activity was increased. Changes in spermidine and spermine levels are minor and cannot be regarded as being significant. By 24 h after anti-IgM treatment, putrescine levels have returned to basal and both growth inhibition and apoptosis are underway (see below). DFMO caused a decrease in all polyamines by 48 h. As will be shown later, this time period also corresponds to a stage of significant DFMO-induced growth inhibition with no apoptosis being observed.

Relationship of ODC to Cell Growth

The experiments presented in Fig. 3 were designed to test whether the growth of Ramos cells is controlled by ODC-related pathways. Cells were treated with DFMO and α-MO (irreversible and reversible inhibitors of ODC, respectively [43]). Cell growth was monitored by two methods, thymidine incorporation to measure DNA replication or a colorimetric assay to determine the number of live cells. Results shown are those for thymidine incorporation. Simultaneous assays for growth using the colorimetric assay were performed with essentially identical results. The effects of ODC inhibition on the cell cycle distribution and on apoptosis are shown later (see Fig. 4).

The first hypothesis evaluated is that ODC activity is essential for the growth of Ramos cells. If this is true, then inhibition of ODC by DFMO or α-MO treatment should cause inhibition of growth. Inhibition of growth by DFMO or α-MO was indeed observed. This can be shown to be due to a decline in putrescine since simultaneous addition of putrescine reduces the extent of growth inhibition. Additional results indicate that when the cells are washed after treatment by the inhibitors and recultured in medium alone, the cells are permitted to grow when the reversible inhibitor α-MO was used but not when the irreversible inhibitor DFMO was used. Furthermore washing the DFMO-treated cells followed by replenishment of putrescine will also reduce the extent of growth inhibition, supporting the view that specific action of DFMO on ODC is involved. These experiments indicate that ODC pathway products such as putrescine, spermine, and spermidine are essential for cell growth in agreement with results reported by others [3–5]. As these experiments were done in the presence of RPMI 1640 medium plus 10% FCS, there is a possibility that endogenous polyamines present in serum may have been partially attenuating some of the DFMO or α-MO effects. Nevertheless, the conclusions reached, namely that ODC inhibition leads to growth inhibition, an effect which is diminished by polyamine supplementation, are still valid.

The second hypothesis tested is whether the anti-IgM-induced growth inhibition also involves the ODC pathway and putrescine production. Anti-IgM causes inhibition of growth and also causes a transient increase in putrescine at 6 to 10 h followed by a later decline (Fig. 2). To mimic this effect, we added putrescine by itself to cells. Only slight growth inhibition was observed relative to that seen with anti-IgM. The inhibition by putrescine is likely due to the high concentra-

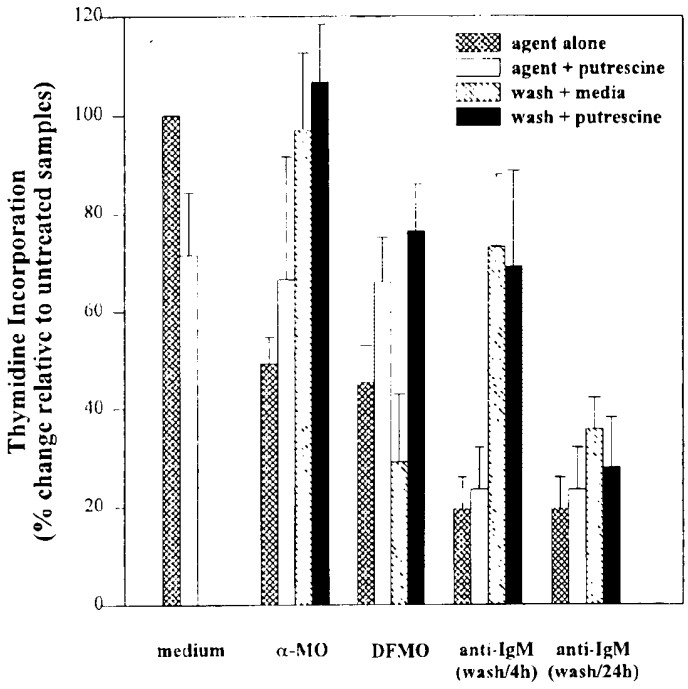

FIG. 3. Growth inhibition caused by anti-IgM or ODC inhibitors. Effects of α-MO, DFMO, or anti-IgM on cell growth were determined. For the growth determination, cells were incubated for 48 h at which time triplicate 200-μl aliquots were transferred into 96-well microtiter plates for thymidine incorporation as described under Materials and Methods. For each set of bars (e.g., α-MO) results are shown for growth in the presence of agent, agent plus 250 μM putrescine, preincubation with agent followed by "wash-out" and continued incubation with either fresh medium or medium containing 250 μM putrescine. Preincubations consisted of either 1 mM α-MO or 1 mM DFMO for 48 h, or 10 μg/ml anti-IgM for either 4 or 24 h. Results of growth in the presence or absence of 250 μM putrescine itself are also presented. "Wash-out" of medium followed by reincubation in medium had no effect and is not shown. Results presented are the means ($\pm$1 SD) from four experiments.

tions and long incubations causing feedback inhibition of ODC. This however does not imply that the anti-IgM-induced inhibition of growth is mediated via putrescine because anti-IgM causes only a transient increase in putrescine levels. Further addition of putrescine to anti-IgM-treated cells giving even higher levels of putrescine has no effect on anti-IgM-induced growth inhibition. Thus putrescine increases are likely not the factor responsible for the anti-IgM-induced growth inhibition. These results imply that the mechanism of anti-IgM-induced inhibition of growth probably is not mediated by putrescine and hence does not involve the ODC pathway.

Inhibition of growth by anti-IgM is conditionally reversible upon washing. The timing of the wash step is critical since washing at 4 h permits some reversal of the growth inhibition but if done at 24 h no reversal of growth inhibition is observed.

Thus inhibition of growth by DFMO is dependent on reduction in polyamines, whereas growth inhibition by anti-IgM is independent of polyamine changes. These results therefore indicate that anti-IgM and DFMO inhibition of growth are achieved through different mechanisms.

Relationship of ODC Pathway to Apoptosis

We have shown that there are two distinct forms of growth inhibition—one achieved by inhibition of the ODC pathway and the other achieved via the B cell antigen receptor (sIg). The experiments in Fig. 4 were designed to test whether inhibition of growth through cell surface receptor and intracellular mechanisms is associated with apoptosis. In these experiments Ramos cells were treated with anti-IgM, DFMO, or polyamines, and then apoptosis was assessed using flow cytometry. For treatments with either spermidine or spermine, the cells had to be in AIM-V medium without serum as the polyamine oxidase activity in fetal calf serum produces toxic acrolein under these conditions [28, 29]. The key findings are as follows: anti-IgM induces apoptosis; the polyamines do not on their own induce apoptosis; none of the polyamines have any effect on apoptosis induced by anti-IgM; DFMO does not induce apoptosis; and DFMO has no effect on the apoptosis induced by anti-IgM. None of the conditions tested, anti-IgM treatment, ODC inhibition, or polyamine supplementation, have any effect on the distribution of cells among the cell cycle phases. Thus growth inhibition seen in Fig. 3 is not accompanied by changes in cell cycle distribution. A change in cell cycle distribution upon washout (see Fig. 3) is also not anticipated. Thus it appears that growth inhibition is accomplished without effects on specific stages of the cell cycle.

Results above (Fig. 3) had shown that the inhibition of growth induced by anti-IgM was independent of the ODC pathway. The current results imply that apoptosis also is unrelated to the ODC pathway. By contrast an equivalent extent of growth inhibition can be produced by the inhibition of ODC using DFMO, and this does not result in apoptosis. The results therefore indicate that two different forms of growth inhibition are present—one initiated via cell surface receptors which results in cell death and is independent of ODC, and the other intracellular and ODC-related which is not connected to cell death.

Effect of TNF-α on Cell Growth

We have previously shown that anti-CD40 and TNF-α inhibit apoptosis induced by anti-IgM [44, 45]. In the experiments presented in Fig. 5, the effects of TNF-α on ODC activity, cell growth, and apoptosis were determined. The results in Fig. 5A show that TNF-α alone is without any effect on ODC activity. Furthermore it has no effect on the transient increase in ODC activity induced by anti-IgM. Figure 5B shows that anti-IgM strongly inhibits growth, as does DFMO, although the

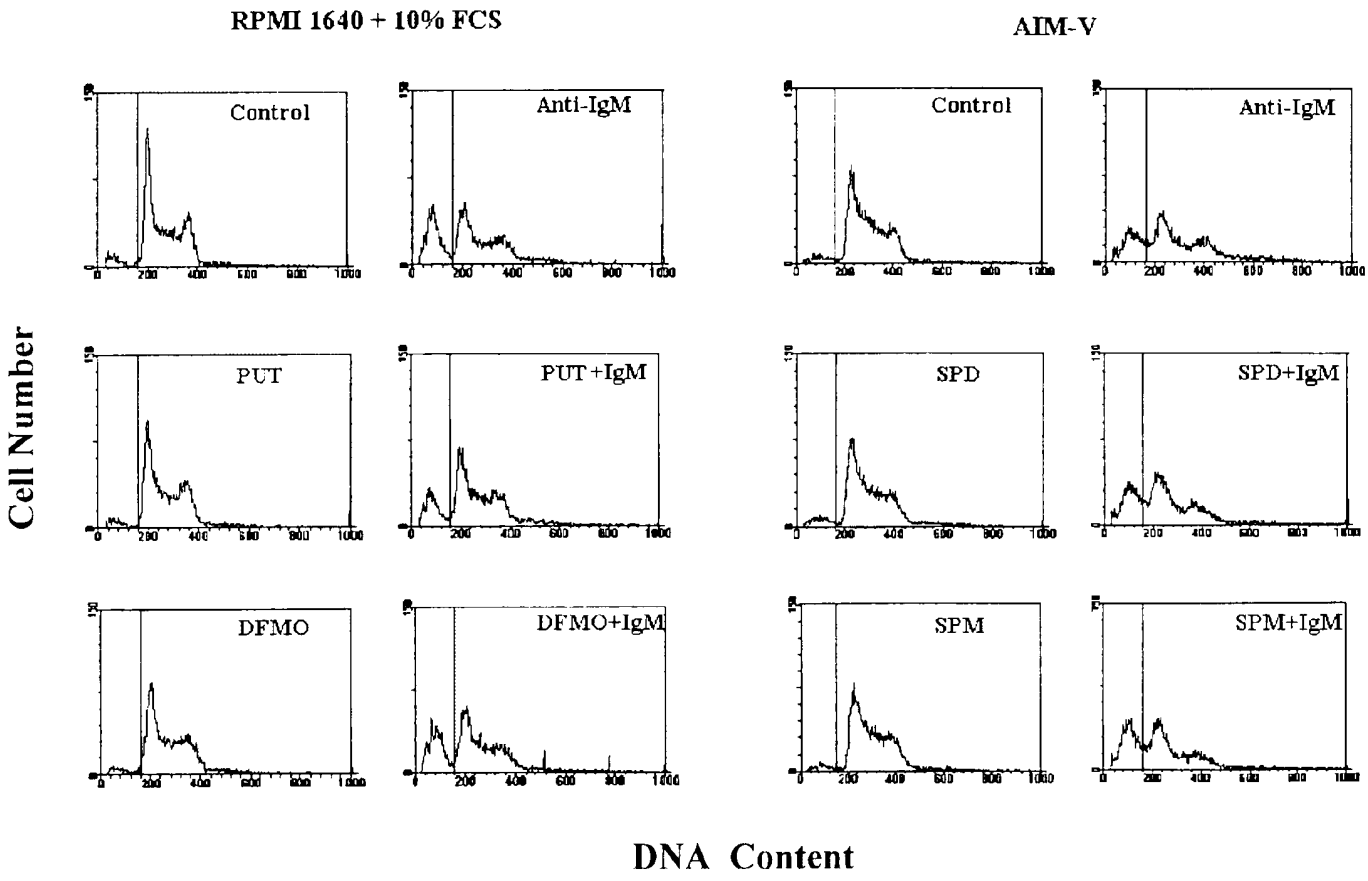

FIG. 4. Effects of anti-IgM, DFMO, and polyamines on apoptosis assessed by flow cytometry. Assessment of apoptosis was performed by flow cytometry. Ramos cells were treated for 24 h with anti-IgM, putrescine, or DFMO in RPMI 1640 medium or with anti-IgM, spermidine, or spermine in AIM-V medium. The cells were then analyzed as described under Materials and Methods. Representative histogram profiles of 10000 cells analyzed using PC-LYSYS program are shown. Untreated cells show bimodal DNA content indicative of the diploid state (G_0/G_1—about channel 200) and the mitotic tetraploid state (G_2/M—about channel 400). S phase cells are intermediate between the G_0/G_1 peak and the G_2/M peak. Apoptosis is manifested by the presence of an A_0 peak with DNA content below that of the G_0/G_1 peak. The vertical line indicates the cutoff point below which all events are considered as being indicative of apoptosis. Abbreviations are as follows: IgM, 10 μg/ml anti-IgM treatment; Put, 250 μM putrescine treatment; DFMO, 1 mM DFMO treatment; SPD, 100 μM spermidine treatment; SPM, 100 μM spermine treatment.

former is a more potent inhibitor. TNF-α alone has no effect on growth and fails to have any effect on the growth inhibition caused by either DFMO or anti-IgM. Figure 5C shows the effect of these agents on apoptosis. Anti-IgM was effective in inducing apoptosis whereas DFMO had no effect. TNF-α did not cause apoptosis by itself or in combination with DFMO; however, it was able to block apoptosis induced by anti-IgM. Results with anti-CD40 were similar to those obtained using TNF-α (unpublished data and [44, 45]).

Regulation of c-myc and ODC

Our previous results indicate that *c-myc* is a key regulator of growth in Ramos cells. Anti-IgM treatment decreased *c-myc* mRNA and protein levels and caused inhibition of growth and apoptosis. Decreases in *c-myc* levels alone however were insufficient for inducing apoptosis in other Burkitt's lymphoma cells. Thus inhibition of *c-myc* was a necessary but insufficient condition for the induction of apoptosis but may be the key event in the inhibition of cell growth [31].

Various investigators have suggested that ODC is a target gene of c-Myc and that Myc protein complexes function as transcriptional activators of the ODC gene [8–10, 46]. In differentiated cells, c-Myc is typically low. Induction of *c-myc* leads to an increase in ODC activity associated with cell growth. In Burkitt's lymphoma cells, *c-myc* is constitutively expressed at high levels. The experiments shown in Fig. 6 were designed to test the involvement of *c-myc* and ODC in the events initiated by anti-IgM and by DFMO, specifically whether a decrease in *c-myc* level would lead to a corresponding decrease in ODC level with its associated cellular effects on growth and apoptosis.

We tested the effect of anti-IgM on *c-myc* mRNA levels and compared these effects to those of ODC

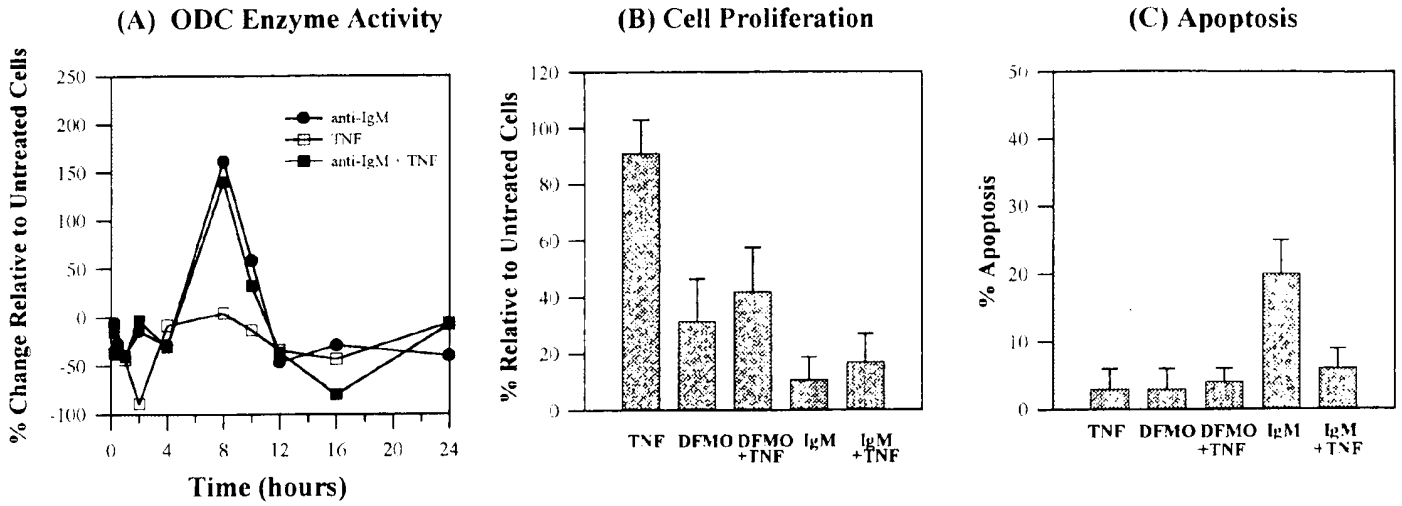

FIG. 5. Effects of TNF-α on anti-IgM and DFMO-treated Ramos cells were evaluated by examining ODC enzyme activity (A), thymidine incorporation (B), and apoptosis (C). Cells were treated with 10 μg/ml anti-IgM, 10 ng/ml TNF-α, or 1 mM DFMO for the indicated times and then assayed as described under Materials and Methods. Panel A shows ODC activities in anti-IgM, TNF-α, and anti-IgM plus TNF-α-treated Ramos cells. Panels B and C show cell growth (thymidine incorporation) at 48 h and apoptosis at 24 h respectively of TNF-α, DFMO, DFMO plus TNF-α, anti-IgM, and anti-IgM plus TNF-α treated cells. In all panels results are presented relative to values obtained in untreated cells incubated for the same time periods. Error bars represent ± 1 SD.

inhibitors. In these experiments cells were treated with either anti-IgM or with DFMO, the two agents mediating the different modes of growth inhibition. The results shown in Fig. 6 indicate that *c-myc* mRNA is decreased significantly by anti-IgM at about 4 h and shows a slow recovery after the point of maximal reduction, similar to our previous results. In this experiment c-Myc protein declines within 4 h and remains depressed at 24 h after anti-IgM treatment as reported previously [31]. Neither DFMO nor putrescine has any effect on *c-myc* levels. Furthermore neither DFMO nor putrescine alters the inhibition of *c-myc* by anti-IgM (unpublished data). By contrast anti-IgM causes an increase in ODC mRNA at about 2 to 6 h and a subsequent decrease at 24 h. Thus the effect of anti-IgM on ODC mRNA is exactly the opposite of what would be expected if c-Myc were a positive regulator of the ODC gene. This implies that ODC mRNA levels are regulated independently of *c-myc*.

DFMO treatment was also observed to cause an increase in ODC mRNA levels. Presumably this is due to the relief of feedback inhibition of putrescine on ODC gene transcription. Putrescine itself has no effect on ODC mRNA levels. Both anti-IgM and DFMO cause a transient increase in ODC mRNA levels. Anti-IgM causes a decrease in *c-myc* whereas neither putrescine augmentation nor putrescine suppression (DFMO addition) has any effect on *c-myc*. These results indicate that *c-myc*, which lies on the growth inhibition pathway for anti-IgM, does not lie on the growth inhibition pathway initiated by DFMO.

DISCUSSION

Role of ODC in Regulation of Cell Growth

The experiments in this paper support a role for ODC in regulation of growth. The evidence includes (a) DFMO, a suicide inhibitor of ODC [40], inhibits growth irreversibly; (b) α-MO, a reversible inhibitor of ODC [43], causes reversible inhibition of growth; and (c) putrescine, the product of ODC action, can reverse DFMO-mediated growth inhibition (Fig. 3). Inhibition of growth can be inferred by cell count or thymidine incorporation or by a colorimetric method and was observed regardless of the method used. These observations are in broad agreement with those of other investigators who have shown that ODC is a key gene in the regulation of cell growth [2–5]. The nature of the role of polyamines in growth remains complex.

Role of ODC in Apoptosis

Treatment of Ramos cells with DFMO or α-MO did not cause induction of apoptosis. This shows that inhibition of growth can be induced without achieving apoptosis, a result further supported by the observation that none of the polyamines, namely putrescine, spermine, and spermidine, induced apoptosis. Neither inhibition of the ODC pathway, reversal of this inhibition, nor supplementation with polyamines induces apoptosis. In addition inhibition of spermidine and spermine synthesis with the *S*-adenosylmethionine decarboxylase (SAM-DC) inhibitor methylglyoxal bis-(guanylhydrazone) also results in growth inhibition without apoptosis (unpublished data).

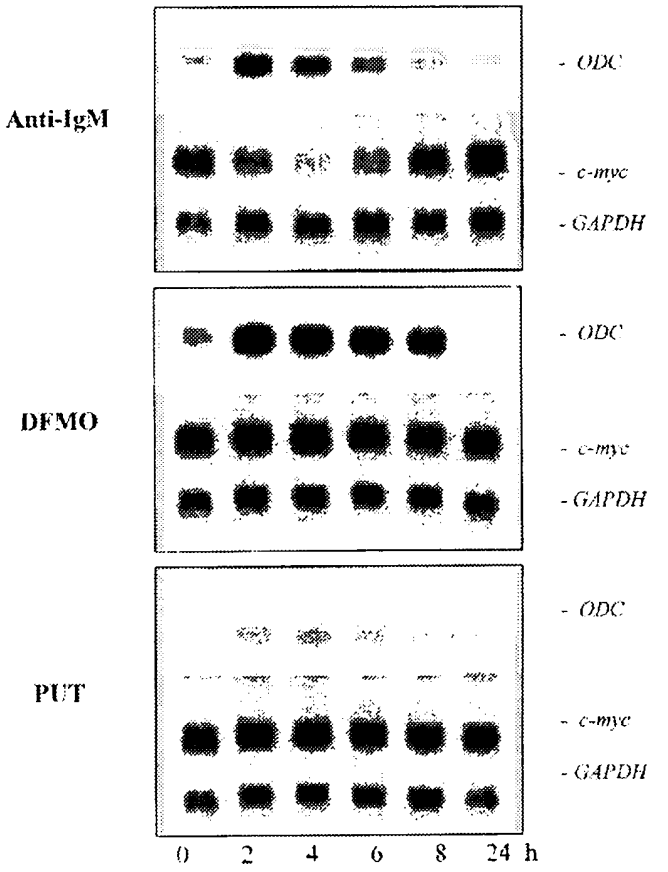

FIG. 6. Effect of anti-IgM, DFMO, and putrescine on *c-myc* and *ODC* mRNA levels. The effects of anti-IgM, DFMO, and putrescine on *c-myc* and *ODC* mRNA levels were evaluated by Northern blotting. Ramos cells were treated with 10 μg/ml anti-IgM, 1 mM DFMO, or 250 μM putrescine for varying times. Total RNA was isolated and Northern blots were performed as described under Materials and Methods.

Our previous observations that inhibition of growth can be achieved without apoptosis are in broad agreement with the experiments discussed above. For example deprivation of growth factors, DMSO treatment of cells [47], low levels of hydroxyurea or aphidicolin,[4] double thymidine block [48], and inhibition of *c-myc* with anti-sense probes [31] all induce growth inhibition without apoptosis.

The significance of this observation is that regulation of growth may lie on a distinct pathway from the regulation of apoptosis. Our previous results had also implied that c-Myc which is expressed at high levels in these cells may be a regulator of growth but is not a contributor to apoptosis in these cell lines.

TNF-α and Anti-CD40

We have previously shown that TNF-α and anti-CD40 are potent inhibitors of anti-IgM-induced apoptosis (Fig. 5C and [44, 45]). Here we have shown that TNF-α has no effect on ODC, does not block the anti-IgM-induced increase in ODC (Fig. 5A), and does not affect inhibition of cell growth by anti-IgM. TNF-α also does not block the inhibition of cell growth induced by DFMO (Fig. 5B). These results suggest that, by using TNF-α (or anti-CD40), the inhibition of apoptosis can be separated from inhibition of growth and these two pathways are likely regulated independently. (See Fig. 7.)

ODC and c-myc

One of the roles postulated for c-Myc-mediated regulation of cell growth is its regulation of ODC. Many studies have indicated that *c-myc* induction causes increases in Myc protein, which in turn switches on the ODC transcription [8, 9, 46, 49–54]. Thus ODC is a target gene for Myc possibly through binding of Myc protein complexes to the E-box domains of the ODC gene [50, 55]. We have previously shown that *c-myc* lies on the pathway to growth inhibition by anti-IgM [31]. We and others [9] therefore reason that if ODC lies on the same pathway, then decreases in c-Myc activity by anti-IgM should lead to decreases in ODC mRNA and possibly of ODC activity as well. Our experiments show the opposite results, namely an increase in ODC mRNA level at 2 to 6 h and an increase in ODC activity at 6 to 10 h instead. These results suggest that the pathways to growth inhibition may be discrete, the anti-IgM pathway involving *c-myc* and possibly other genes, while the ODC pathway may be unrelated to *c-myc* altogether [56].

We have also examined the reverse proposition namely that ODC, and by implication polyamines, may also regulate *c-myc* mRNA levels. ODC inhibitors have no effect on *c-myc* expression although they inhibit cell growth. This is true regardless of whether reversible or irreversible inhibitors are used. Putrescine is also without effect on *c-myc*. These results lend further support to distinct pathways regulating growth inhibition, one via cell surface receptors such as the B cell antigen receptor (anti-IgM treatment) and the other via metabolic means within the cells (ODC effect).

Anti-IgM-Mediated Growth Inhibition Does Not Involve ODC

In spite of ODC not lying on the pathway to apoptosis, the question still arises whether the variety of signaling pathways triggered by anti-IgM treatment of B cells contributes to the regulation of ODC activity. The results showed that ODC activity and intracellular levels of putrescine are transiently (6 to 10 h) increased by anti-IgM treatment. The specific signaling pathway involved in the induction is not clear. Induction of ODC by anti-IgM appears to be self-limited due to feedback regulation by the downstream products such as putrescine [6, 29, 41, 42]. In Fig. 1 we have shown that putres-

[4] C.-K. E. Lin *et al.*, Apoptosis may be either suppressed or enhanced with strategic combinations of antineoplastic drugs or anti-IgM (paper submitted for publication).

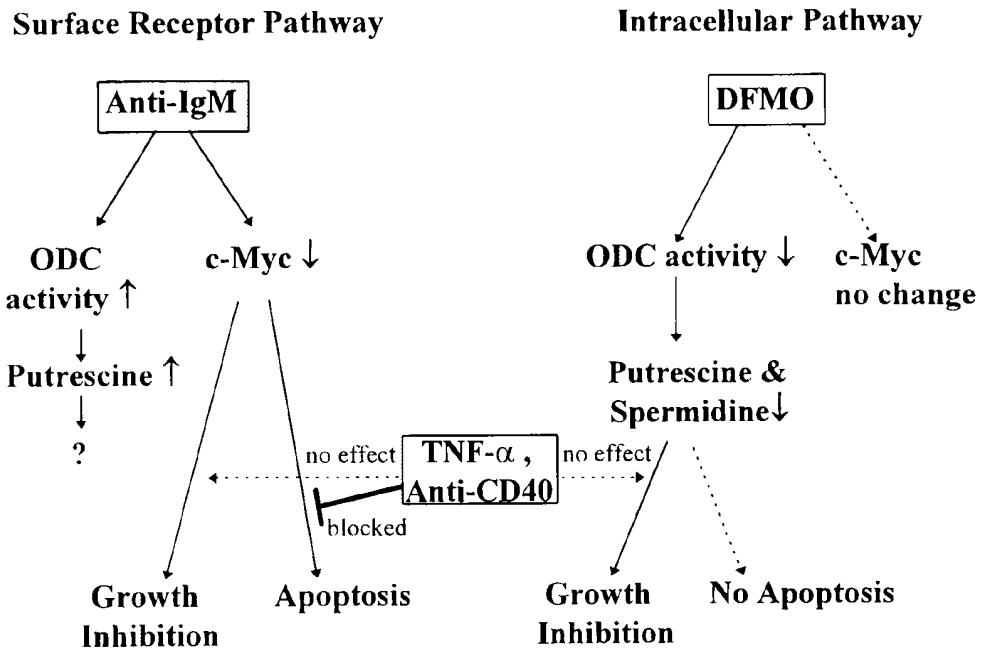

FIG. 7. Multiple pathways to growth inhibition and apoptosis are present in Burkitt's lymphoma cell lines. Stimulation of the B cell surface receptor of Ramos cells using anti-IgM results in a transient increase in ODC activity and putrescine levels, along with a decline in c-Myc. The effect of increased ODC and putrescine is not clear. *c-myc* down-regulation is found to be correlated with growth inhibition and apoptosis. On the other hand, inhibition of ODC using DFMO results in growth inhibition without apoptosis. *c-myc* is unaffected by this treatment. Thus *c-myc*-dependent and -independent growth inhibition can be achieved using anti-IgM or DFMO, respectively. TNF-α or anti-CD40 prevent anti-IgM-induced apoptosis but show no effect on blocking growth inhibition in either pathway. This suggests growth inhibition can be separated from apoptosis in this cell model.

cine itself inhibits the anti-IgM-mediated induction of ODC activity.

Both anti-IgM and ODC inhibitors induce growth inhibition. The question therefore arises whether the growth inhibition induced by these two is similar or different. Our results indicate that growth inhibition occurring through mechanisms utilizing ODC induction can be reversed by the addition of putrescine. Growth inhibition induced by anti-IgM however cannot be blocked by the addition of putrescine. The results suggest that the pathway leading to growth inhibition from anti-IgM is not mediated by ODC and is thus distinct from those in which ODC plays a key role. "Wash-out" experiments imply that a continued presence of anti-IgM at the B cell antigen receptor may be required for the induction of growth inhibition. The distinct pathways of growth inhibition are summarized in the model shown in Fig. 7.

Anti-IgM-Mediated Growth Inhibition Involves c-Myc Protein

What is the relationship of the observed down-regulation of *c-myc* to the commitment points for anti-IgM-induced growth arrest and apoptosis? When does the cell decide between growth arrest and apoptosis vs. recovery and what are the biochemical events associated with this commitment point? Our results strongly imply that in Burkitt's lymphoma cells the decline in c-Myc protein is a key event in growth inhibition and apoptosis [31]. The time at which this pathway becomes irreversible is not clear. The commitment to growth arrest following anti-IgM treatment is associated with decline in c-Myc levels. The evidence is as follows: (a) The initial event is a decline in *c-myc* mRNA levels accompanied by a decline in protein levels. Although the mRNA levels are observed to recover, the protein levels do not recover. (b) Antisense *c-myc* oligodeoxynucleotide addition causes growth arrest with or without apoptosis [31]. (c) Cells can be arrested for growth without apoptosis under a variety of conditions [31, 48]. Under these conditions we find that c-Myc protein decreases. We have developed a model (see Fig. 11 in [31]) which relates growth arrest to apoptosis. In this model the p53 mutant status of the cell forces the cell to grow even under conditions where growth arrest is mandated by other factors. This compulsion to grow under conditions which are not propitious for growth leads the cells to apoptosis [57, 58]. The wash-out experiments presented here suggest that growth inhibition can be prevented by curtailing signal development, transduction factor activation, or transcription factor activation. The precise events involved are not clear.

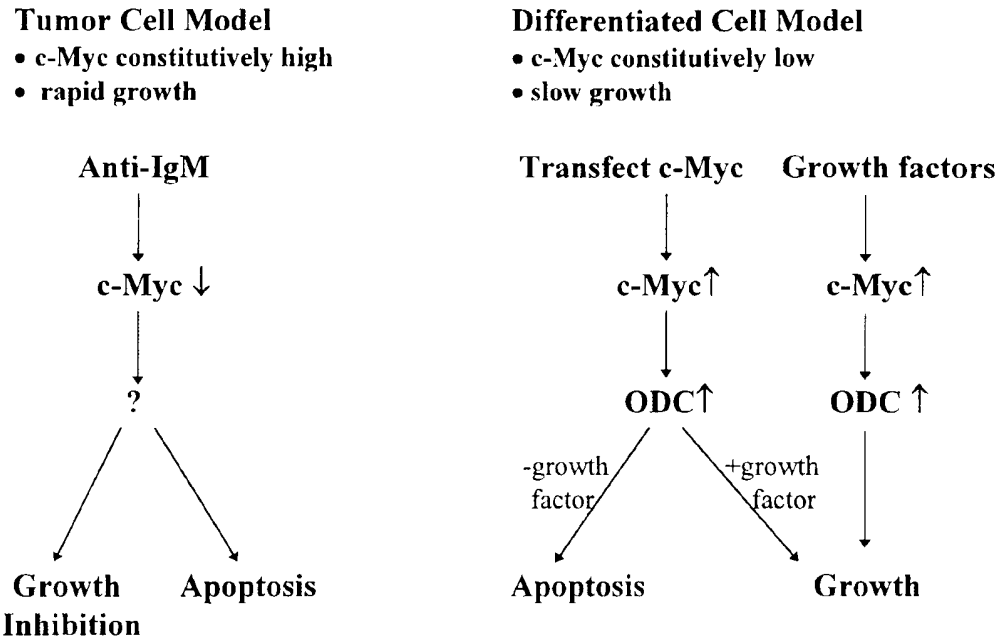

FIG. 8. Contrasting role of c-Myc in tumor and differentiated cells. The role of c-Myc in the regulation of growth and apoptosis depends on the cell system investigated. In rapidly growing tumor cells (such as Ramos), c-Myc is constitutively expressed at high levels. Activation of this cell system (i.e., crosslinking with anti-IgM) will result in down-regulation of *c-myc* and consequent growth inhibition or apoptosis. Although the mechanisms are still unknown, these pathways appear to be independent of ODC activity. In systems where c-Myc is normally expressed at low basal levels (e.g., differentiated cells, HL-60) growth is retarded. Forced expression of *c-myc* by plasmid transfection or stimulation with hormone or growth factors induces c-Myc which in turn up-regulates ODC activity, stimulates polyamine production, and induces cell growth. Depletion of growth factors under conditions where *c-myc*-driven growth pathways are initiated may instead direct the cells toward apoptosis. This model represents a composite view derived from the published works of various groups [31, 32, 57–59].

CONCLUSIONS

In this report a specific hypothesis has been examined: In cells with constitutively high *c-myc* activity, does a decline in *c-myc* activity caused by cell surface receptors result in a decrease in ODC activity? Is this decrease associated with either growth arrest or apoptosis? Specifically we examined whether in B cells the anti-IgM-induced growth inhibition and apoptosis are mediated by the involvement of ODC (Fig. 8). This hypothesis is valid in that growth induction in some cell systems is mediated by an induction of c-Myc acting on ODC, and by our previous report that c-Myc regulation is a key step in anti-IgM-mediated growth inhibition and apoptosis induction. The hypothesis has been broken down into various components: (a) whether direct ODC inhibition or the products of ODC action cause either growth arrest or apoptosis, and (b) since c-Myc has been reported to be an inducer of ODC, whether the converse situation also holds (i.e. whether anti-IgM-induced decline in *c-myc* activity is accompanied by a decline in ODC activity).

Our results indicate that a decline in ODC due to ODC inhibitors results in growth inhibition without apoptosis. We find that anti-IgM which potently down-regulates *c-myc* does not down-regulate ODC which is paradoxically transiently increased. Anti-IgM treatment however does result in both growth inhibition and induction of apoptosis. Thus ODC is not a key mediator of anti-IgM-induced pathways regulating either growth arrest or apoptosis. The mechanism of growth inhibition due to anti-IgM is distinct from that of ODC inhibitor-mediated growth inhibition. Multiple pathways may contribute to growth regulation of which ODC may be selectively involved. Overall the results imply that while up-regulation of *c-myc* from low levels may augment ODC and lead to growth, the converse model is not correct, namely down-regulation of *c-myc*, in cells that overexpress it, does not affect ODC and in these systems ODC is not on the pathway from *c-myc* to apoptosis. Thus the context of *c-myc* regulation (constitutive or inducible) may have distinct consequences for the involvement of ODC and its contribution to growth or apoptosis.

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